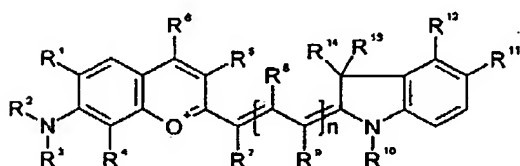
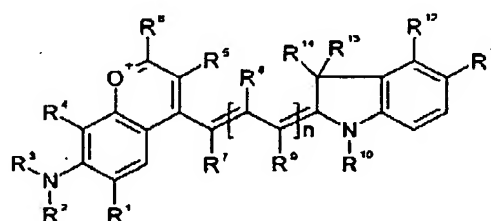


**Benzopyrro-polymethine-based hydrophilic markers**

The invention is concerned with benzopyrro-polymethine-based markers for use in optical, and in particular optical-fluorescent, determination and detection procedures, for example in medicine, the pharmaceutical industry and in bioscience, materials science and environmental science.

The challenge was to create highly hydrophilic content polymethine-based fluorescent markers with high extinction coefficients and a high degree of photo and storage stability. These can be excited simply to emit fluorescent light by monochromatic (laser/laser diodes) or polychromatic light (white light sources) in the UV, visual or NIR spectrum range or can function as quenchers.

For the purposes of the invention, general formula I and/or II polymethine-based chromophores are used.

**I****II**

The invention covers the use as markers of appropriate asymmetrical polymethine-based hydrophilic complexes and their application in optical and in particular optical fluorescent, determination and detection procedures.

Typical applications are based on the reaction of dye-marked biomolecules such as, for example, antigens, antibodies or DNA segments, in each case with the complementary species, enabling, inter alia, enzyme kinetics, receptor-ligand interactions and nucleic acid hybridization kinetics to be measured both *in vitro* and *in vivo*. Furthermore, the markers are interesting from the point of view of the pharmacological characterization of receptors or active materials.

Accordingly, opportunities for their use exist in, for example, medicine and the pharmaceutical industry, bioscience, materials science, environmental monitoring and the detection of organic and inorganic micro-specimens occurring in nature and the world of technology and in other areas as well.

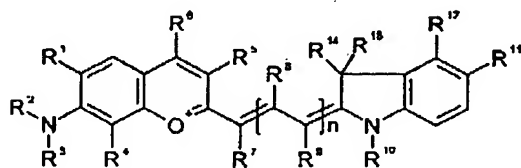
Symmetrical xanthylium salt (fluoresceine and rhodamine) or polymethine (indocyanine) as claimed in, for example, US patent specification 5 627 027 are normally used.

All these markers have the disadvantage that they tend towards aggregation and dimerization owing to the planarity of the  $\pi$ -electron system, especially in aqueous systems. Moreover, insufficiently hydrophilic markers show non-specific interactions with different surfaces, resulting in problems with cleaning the corresponding conjugates and in an unsatisfactory signal/noise ratio.

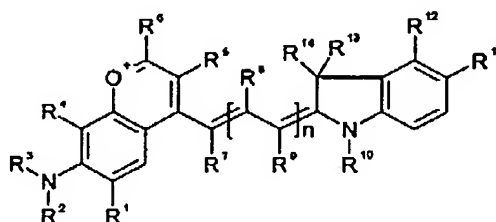
In order to circumvent these disadvantages, corresponding asymmetrical polymethines on the basis of benzob[pyran-2-ylidene or benzo[b]pyran-4-ylidene – compounds were described in patent specifications PCT/DE 00/00802 and PCT/DE 01/01946.

We were then able to improve these markers even further by introducing additional substituents which increased the level of marker hydrophily.

New general formula I and/or II polymethine-based hydrophil markers are now the subject of the invention,



I



II

where

- $R^1 - R^{14}$  are the same or different and may be hydrogen, alkyl-, *tert*-alkyl, aryl-, carboxyaryl-, dicarboxyaryl, heteroaryl-, cycloalkyl-, heterocycloalkyl-, alkyloxy-, alkylmercapto- (with "alkyl" and "cycloalkyl" also including olefin linkage residues), aryloxy-, arylmercapto-, heteroaryloxy-, heteroarylmercapto-, hydroxy-, nitro- or cyano residues and  $R^1$  and  $R^2$ ,  $R^2$  and  $R^3$ ,  $R^3$  and  $R^4$ ,  $R^5$  and  $R^7$ ,  $R^9$  and  $R^{10}$ ,  $R^{11}$  and  $R^{12}$  or  $R^{12}$  and  $R^{13}$  may form one or more aliphatic, heteroaliphatic or aromatic rings,
- at least one or more of the  $R^1 - R^{14}$  substituents may constitute solubilizing or ionizing or ionized substituents such as  $SO_3^-$ ,  $PO_3^{2-}$ ,  $CO_2H$ ,  $OH$ ,  $NR_3^+$ , cyclodextrines or sugars, which determine the hydrophil characteristics of dyes; these substituents may also be linked to the actual basic chromophore by means of an aliphatic or heteroaliphatic or cyclical spacer group, as the case may be,
- at least one of the  $R^1 - R^{14}$  substituents stands for a reactive group of the isocyanate, isothiocyanate, hydrazine, amin, mono- and dichlor- or mono- and dibromotriazine, aziridine, sulfonylhalogenide, *N*-hydroxysuccinimide, imido-ester, glyoxal or aldehyde or maleimide or iodacetamide and phosphoramidite type; the substituent in question may be linked to the actual basic chromophore by means of an aliphatic or heteroaliphatic or cyclical spacer group, as the case may be,

- the aliphatic or heteroaliphatic spacer group consists of a structural element -  $[(CH_2)_a-Y-(CH_2)_b]_c$ , in which Y - the same or different - may be a  $CR_2$ -, O-, S-,  $SO_2$ ,  $SO_2NH$ -,  $NR$ -,  $COO$ - or  $CONR$  function, with R assuming the functions of  $R^1$  -  $R^{14}$  and a and b - the same or different - representing values 0 - 18 and c values 1- 18,
- n stands for numerical values 0, 1, 2 or 3; substituents  $R^8$  and  $R^9$  - doubled or threefold to give n = 2 or 3 respectively – may be the same or different.

The complexes covered by the invention ("invention complexes") may be used as dyes for the optical marking of proteins, nucleic acids, oligomers, DNA, RNA, biological cells, lipids, mono-, oligo- and polysaccharids, ligands, receptors, polymers, pharmaceutical or polymer particles and coupled via the functional groups to an  $HO$ -,  $H_2N$ -,  $HS$  or  $HO_2C$  function of the substances to be determined, as dyes used in systems determining the quality or quantity of proteins, nucleic acids, oligomers, DNA, RNA, biological cells, lipids, polymers, pharmaceutical or polymer particles.

This coupling reaction is achieved to advantage in organic or aqueous solutions.

The conjugates from the invention complexes and biomolecules display fluorescing characteristics or deactivate the activated state without emitting light (quencher).

The invention complexes have an application in qualitative and quantitative optical and in particular optical fluorescent, determination procedures, including immune tests, hybridization procedures, chromatographic or electrophoretic procedures, FRET systems and high-throughput screening or for the analysis of receptor-ligand change effects on a microarray.

General formula I and/or II polymethines may be used as dyes for optical marking of organic or inorganic identification units, such as, for example, aminoacids, peptides, proteins, antigens, haptens, enzyme substrates, enzyme co-factors, biotins, carotinoids, hormones, neuro-hormones, neuro-transmitters, growth factors,

lympholocines, lectins, toxins, carbohydrates, oligosaccharides, polysaccharides, dextrans, nucleic acids, oligonucleotides, DNA, RNA, biological cells, lipids, receptor-linking pharmaceutical or organic or inorganic polymer carriers.

Marking of identification units can be done by the the formation of ionic interactions occurring between the general formula I and/or II complexes and the materials to be marked.

The identification unit or carrier can also be linked covalently with the fluorophore. This coupling reaction can be achieved in an aqueous or mainly aqueous solution, preferably at room temperature. The resulting probe (conjugate) determines the quality or quantity of different bio-materials or other organic and inorganic materials through the use of optical procedures.

Both general formula I and/or II complexes and derived systems can be used in qualitative and quantitative optical, and in particular optical fluorescent, determination procedures for diagnosing cell characteristics (molecular imaging), biosensors (*point of care* measurements), genome research and miniaturization technologies. Typical applications occur in cytometry and cell sorting, fluorescence correlation spectroscopy (FCS), *Ultra-High-Throughput-Screening* (UHTS), *multicolor fluorescence in-situ* hybridization (FISH), FRET systems and microarrays (DNA- and protein chips).

A microarray is a grid arrangement of molecules immobilized on at least one surface which can be used for the study of receptor-ligand change effects. A grid arrangement denotes more than two differing surface molecules which are immobilized in known positions in varying pre-defined regions of the surface.

A receptor is a molecule which has an affinity to a given ligand. Receptors can be naturally occurring or synthetically produced molecules. They can be used in pure form or in association with another species. They can be linked covalently or non-covalently to a linkage partner either directly or by means of certain coupling mediators.

Examples of receptors which are detectable on the basis of this invention include agonists and antagonists for cell membrane receptors, toxins and other poisonous substances, viral epitopes, hormones such as opiates and steroids, hormone receptors, peptides, enzymes, enzyme substrates, active substances acting as co-factors, lectines, sugars, oligonucleotides, nucleic acids, oligosaccharides, cells, cell fragments, tissue fragments, proteins and antibodies. However, they are not limited to these substances.

A ligand is a molecule which is recognized by a certain receptor. Examples of ligands which are detectable by means of the invention compounds include agonists and antagonists for cell membrane receptors, toxins and other poisonous substances, viral epitopes, hormones such as opiates and steroids, hormone receptors, peptides, enzymes, enzyme substrates, active substances acting as co-factors, lectines, sugars, oligonucleotides, nucleic acids, oligosaccharides, proteins and antibodies. However, they are not limited to these substances.

The following advantages are achieved by the preparation of asymmetrical polymethines possessing, on the one hand, an easily derivatizable heterocycle selected from CH acidic compounds as a terminal function and, on the other, a 6 ring heterocycle (novel substitution).

Already relatively small molecules absorb in the spectral range over 550 nm and display fundamentally improved photochemical and thermal stability compared with previously known polymethines with maximum absorption maxima above 650 nm (penta- and heptamethines).

It is possible, through *molecular engineering*, to control the position and intensity of absorption and emission maxima at will and to adjust them in line with emission wavelengths of different activating lasers, in particular diode lasers.

The invention complexes are comparatively simply to manufacture by condensing the two different CH-acid heterocycles and a C-1, C-3 or C-5 component ("mulligan procedure").

Further manufacturing procedures involve condensation of one of the CH acid heterocycles with the C-1, C-3 or C-5 component at an initial reaction stage and – following the isolation of the 1:1 condensation product – conversion to a polymethine with the second CH-acid heterocycle in a subsequent condensation process. The sequence of heterocycle applications involved is not inconsiderable. On the basis thereof, many heavily hydrophilic, variously functionalized dyes differing in respect of total charge and the specificity/reactivity of the activated groups used for the purposes of immobilization can be easily manufactured in few reaction steps.

The examples shown below in the drawing should provide a more detailed explanation of the invention.

- Fig. 1:      **Synthesis of DY - 631**  
              **Example 1**
- Fig. 2:      **Synthesis of DY-631 N-Hydroxysuccinimidyl ester**  
              **Example 2**
- Fig. 3:      **Synthesis of DY - 636**  
              **Example 3**
- Fig. 4:      **Synthesis of DY – 636 N-Hydroxysuccinimidyl ester**  
              **Example 4**
- Fig. 5:      **Synthesis of DY - 651**  
              **Example 5**
- Fig. 6:      **Synthesis of DY – 651 N-Hydroxysuccinimidyl ester**  
              **Example 6**
- Fig. 7:      **Synthesis of DYQ - 661**  
              **Example 7**
- Fig. 8:      **Synthesis of DYQ – 661 N-Hydroxysuccinimidyl ester**  
              **Example 8**
- Fig. 9:      **Synthesis of DY - 676**  
              **Example 9**
- Fig. 10:     **Synthesis of DY – 676 N-Hydroxysuccinimidyl ester**  
              **Example 10**
- Fig. 11:     **Synthesis of DY - 731**  
              **Example 11**

- Fig. 12: Synthesis of DY – 731 N-Hydroxysuccinimidyl ester  
Example 12
- Fig. 13: Synthesis of DY - 751  
Example 13
- Fig. 14: Synthesis of DY – 751 N-Hydroxysuccinimidyl ester  
Example 14
- Fig. 15: Synthesis of DY - 776  
Example 15
- Fig. 16: Synthesis of DY – 776 N-Hydroxysuccinimidyl ester  
Example 16
- Fig. 17: Synthesis of DY - 681  
Example 17
- Fig. 18: Synthesis of DY – 681 N-Hydroxysuccinimidyl ester  
Example 18
- Fig. 19: Synthesis of DY - 701  
Example 19
- Fig. 20: Synthesis of DY – 701 N-Hydroxysuccinimidyl ester  
Example 20
- Fig. 21: Composition of DY - 781  
Example 21
- Fig. 22: Synthesis of DY – 781 N-Hydroxysuccinimidyl ester  
Example 22
- Fig. 23: UV-vis spectrum of DY-630 and DY-631 streptavidine conjugate
- Fig. 24: UV-vis spectrum of DY-700 and DY-701 avidine conjugate

The concept of "vacuum" below refers to the 30 – 150 mbar pressure range. Liquid mixing ratios are by volume. RT denotes room temperature. NHS means N-Hydroxysuccinimide; DCC means Dicyclohexylcarbodiimide; DMF means N,N-Dimethylformamide.

**Examples 1 – 16 (general formula II complexes)**



### 1. Synthesis of DY - 631

180 mg (0.5 mmol) 2-*tert*-butyl-7-diethylamino-4-methyl-chromenylium-tetrafluoroborate and 242 mg (0.5 mmol) 3-(3-ethoxycarbonylpropyl)-2,3-dimethyl-5-sulfonato-1-(3-sulfonatopropyl)-3*H*-indolium sodium salt are dissolved in 50 ml of acetanhydride, with 75  $\mu$ l (0.6 mmol) of trimethylorthoformate and 1 ml of pyridine. The solution is stirred for approx. 30 min at approx. 140 °C. After cooling to RT, the solvent is removed in the vacuum.

The residue is heated to reflux for 2 hours in a mixture of 10 ml of acetone and 10 ml of 2 M hydrochloric acid, the reaction solution neutralized with NaHCO<sub>3</sub> and the solvent distilled in the vacuum. The residue is chromatographed (SiO<sub>2</sub>-RP-18, eluent methanol/ water - 6:4).

145 mg (39 %) yield - UV/Vis (ethanol)  $\lambda_{\text{max}}$  ( $\epsilon$ ) = 637 nm (185.000 l·mol<sup>-1</sup>·cm<sup>-1</sup>). - fluorescence  $\lambda_{\text{em}}$  = 658 nm. - MS (ESI): 713.2 [M]<sup>+</sup>; 356.4 [M - H]<sup>2+</sup>. - C<sub>36</sub>H<sub>45</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>Na (736.88).

### 2. Synthesis of DY – 631 N-Hydroxysuccinimidyl ester

15 mg DY - 631, 14 mg DCC, 4 mg NHS and 10  $\mu$ l pyridine are dissolved in 2 ml of DMF and stirred at RT for 24 h. The solvent is removed in vacuum. The residue is washed with diethylether and dried in vacuum. The reaction is quantitative.

### 3. Synthesis of DY - 636

206 mg (0.5 mmol) 10-*tert*-butyl-8-methyl-2,3,5,6-tetrahydro-1*H*,4*H*-11-oxonia-3a-aza-benzo[*de*]anthracene-tetrafluoroborate and 242 mg (0.5 mmol) 3-(3-ethoxycarbonylpropyl)-2,3-dimethyl-5-sulfonato-1-(3-sulfonatopropyl)-3*H*-indolium sodium salt are reacted and processed in accordance with example 1.

135 mg (36 %) yield - UV/Vis (ethanol)  $\lambda_{\text{max}}$  ( $\epsilon$ ) = 645 nm (155.000 l·mol<sup>-1</sup>·cm<sup>-1</sup>). - fluorescence  $\lambda_{\text{em}}$  = 670 nm. - MS (ESI): 737.1 [M]<sup>+</sup>; 368.4 [M-H]<sup>2+</sup>. - C<sub>38</sub>H<sub>45</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>Na (760.91).

### 4. Synthesis of DY – 636 N-Hydroxysuccinimidyl ester

15 mg DY - 636, 14 mg DCC and 4 mg NHS are reacted and processed in accordance with example 2.

### 5. Synthesis of DY - 651

206 mg (0.5 mmol) 2-*tert*-butyl-8-ethyl-4,5,7,7-tetramethyl-7,8-dihydro-1-oxonia-8-aza-anthracene-tetrafluoroborate and 242 mg (0.5 mmol) 3-(3-ethoxycarbonylpropyl)-

2,3-dimethyl-5-sulfonato-1-(3-sulfonatopropyl)-3*H*-indolium sodium salt are reacted and processed in accordance with example 1.

145 mg (38 %) yield - UV/Vis (Ethanol)  $\lambda_{\text{max}}$  ( $\epsilon$ ) = 653 nm (160.000 l·mol<sup>-1</sup>·cm<sup>-1</sup>). - fluorescence  $\lambda_{\text{em}}$  = 678 nm. - MS (ESI<sup>+</sup>): 765.1 [M]<sup>+</sup>; 382.4 [M-H]<sup>2+</sup>. - C<sub>40</sub>H<sub>49</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>Na (888.96).

#### 6. Synthesis of DY – 651 N-Hydroxysuccinimidyl ester

15 mg DY - 651, 14 mg DCC and 4 mg NHS are reacted and processed in accordance with example 2.

#### 7. Synthesis of DYQ - 661

196 mg (0.5 mmol) 7-diethylamino-3,4-dimethyl-2-phenyl-chromenylium-tetrafluoroborate and 242 mg (0.5 mmol) 3-(3-ethoxycarbonylpropyl)-2,3-dimethyl-5-sulfonato-1-(3-sulfonatopropyl)-3*H*-indolium sodium salt are reacted and processed in accordance with example 1.

145 mg (37 %) yield - UV/Vis (Ethanol)  $\lambda_{\text{max}}$  ( $\epsilon$ ) = 661 nm (116.000 l·mol<sup>-1</sup>·cm<sup>-1</sup>). - MS (ESI<sup>+</sup>): 747.1 [M]<sup>+</sup>, 373.6 [M-H]<sup>2+</sup>. - C<sub>39</sub>H<sub>43</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>Na (770.90).

#### 8. Synthesis of DYQ – 661 N-Hydroxysuccinimidyl ester

15 mg DYQ - 661, 14 mg DCC and 4 mg NHS are reacted and processed in accordance with example 2.

#### 9. Synthesis of DY - 676

216 mg (0.5 mmol) 8-ethyl-4,5,7,7-tetramethyl-2-phenyl-7,8-dihydro-1-oxonia-8-aza-anthracene-tetrafluoroborate and 242 mg (0.5 mmol) 3-(3-ethoxycarbonylpropyl)-2,3-dimethyl-5-sulfonato-1-(3-sulfonatopropyl)-3*H*-indolium sodium salt are reacted and processed in accordance with example 1.

150 mg (37 %) yield - UV/Vis (Ethanol)  $\lambda_{\text{max}}$  ( $\epsilon$ ) = 674 nm (84.000 l·mol<sup>-1</sup>·cm<sup>-1</sup>). - fluorescence  $\lambda_{\text{em}}$  = 699 nm. - MS (ESI<sup>+</sup>): 785.5 [M]<sup>+</sup>. - C<sub>42</sub>H<sub>45</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>Na (807.95).

#### 10. Synthesis of DY – 676 N-Hydroxysuccinimidyl ester

15 mg DY - 676, 14 mg DCC and 4 mg NHS are reacted and processed in accordance with example 2.

#### 11. Synthesis of DY - 731

180 mg (0.5 mmol) 2-*tert* butyl-7-diethylamino-4-methyl-chromenylium-tetrafluoroborate und 307 mg (0.5 mmol) 3-(3-ethoxycarbonylpropyl)-3-methyl-2-(4-phenyl-aminobuta-1,3-dienyl)-5-sulfonato-1-(3-sulfonatopropyl)-3*H*-indolium sodium salt are dissolved in 50 ml of acetanhydride with 1 ml of pyridine. The solution is stirred for approx. 30 min at approx. 140 °C. After cooling to RT, the solvent is removed in the vacuum.

The residue is refluxed for 2 hours in a mixture of 10 ml acetone and 10 ml 2-M hydrochloric acid, the reaction solution neutralized with NaHCO<sub>3</sub> and the solvent distilled in the vacuum. The residue is chromatographed (SiO<sub>2</sub>-RP-18, eluent methanol/ water - 6:4).

120 mg (31 %) yield - UV/Vis (ethanol)  $\lambda_{\max} (\epsilon) = 736 \text{ nm} (225.000 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1})$ . - fluorescence  $\lambda_{\text{em}} = 759 \text{ nm}$ . - MS (ESI<sup>+</sup>): 739.2 [M]<sup>+</sup>, 369.5 [M - H]<sup>2+</sup>. - C<sub>38</sub>H<sub>47</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>Na (762.92).

#### 12. Synthesis of DY – 731 N-Hydroxysuccinimidyl ester

15 mg DY - 731, 14 mg DCC and 4 mg NHS are reacted and processed in accordance with example 2.

#### 13. Synthesis of DY - 751

206 mg (0.5 mmol) 2-*tert*-butyl-8-ethyl-4,5,7,7-tetramethyl-7,8-dihydro-1-oxonia-8-aza-anthracene-tetrafluoroborate and 307 mg (0.5 mmol) 3-(3-ethoxycarbonylpropyl)-3-methyl-2-(4-phenyl-aminobuta-1,3-dienyl)-5-sulfonato-1-(3-sulfonatopropyl)-3*H*-indolium sodium salt are reacted and processed in accordance with example 11.

120 mg (29 %) yield - UV/Vis (ethanol)  $\lambda_{\max} (\epsilon) = 751 \text{ nm} (220.000 \text{ l} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1})$ . - fluorescence  $\lambda_{\text{em}} = 779 \text{ nm}$ . - MS (ESI<sup>+</sup>): 793.1 [M + H]<sup>+</sup>, 419.4 [M + 2 Na]<sup>2+</sup>, 408.4 [M + H + Na]<sup>2+</sup>, 397.4 [M + 2 H]<sup>2+</sup>. - C<sub>42</sub>H<sub>51</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>Na (814.99).

#### 14. Synthesis of DY – 751 N-Hydroxysuccinimidyl ester

15 mg DY - 751, 14 mg DCC and 4 mg NHS are reacted and processed in accordance with example 2.

#### 15. Synthesis of DY - 776

216 mg (0.5 mmol) 8-ethyl-4,5,7,7-tetramethyl-2-phenyl-7,8-dihydro-1-oxonia-8-aza-anthracene-tetrafluoroborate and 307 mg (0.5 mmol) 3-(3-ethoxycarbonylpropyl)-3-

methyl-2-(4-phenylamino-buta-1,3-dienyl)-5-sulfonato-1-(3-sulfonatopropyl)-3*H*-indolium sodium salt are reacted and processed in accordance with example 11. 110 mg (26 %) yield - UV/Vis (Ethanol)  $\lambda_{\text{max}}$  ( $\epsilon$ ) = 771 nm (147.000 l·mol<sup>-1</sup>·cm<sup>-1</sup>). - fluorescence  $\lambda_{\text{em}}$  = 801 nm. - MS (ESI<sup>+</sup>): 813.1 [M + H]<sup>+</sup>, 429.2 [M + 2 Na]<sup>2+</sup>, 418.3 [M + H + Na]<sup>2+</sup>, 407.3 [M + 2 H]<sup>2+</sup>. - C<sub>44</sub>H<sub>47</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>Na (834.98).

#### 16. Synthesis of DY – 776 N-Hydroxysuccinimidyl ester

15 mg DY - 776, 14 mg DCC and 4 mg NHS are reacted and processed in accordance with example 2.

#### Examples 17 – 22 (general formula I complexes)

##### 17. Synthesis of DY - 681

180 mg (0.5 mmol) 4-*tert*-butyl-7-diethylamino-2-methyl-chromenylium-tetrafluoroborate and 242 mg (0.5 mmol) 3-(3-ethoxycarbonylpropyl)-2,3-dimethyl-5-sulfonato-1-(3-sulfonatopropyl)-3*H*-indolium sodium salt are reacted and processed in accordance with example 1.

140 mg (39 %) yield - UV/Vis (Ethanol)  $\lambda_{\text{max}}$  ( $\epsilon$ ) = 691 nm (125.000 l·mol<sup>-1</sup>·cm<sup>-1</sup>). - fluorescence  $\lambda_{\text{em}}$  = 708 nm. - MS (ESI<sup>+</sup>): 713.2 [M]<sup>+</sup>; 356.4 [M - H]<sup>2+</sup>. - C<sub>36</sub>H<sub>45</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>Na (736.88).

##### 18. Synthesis of DY – 681 N-Hydroxysuccinimidyl ester

15 mg DY - 681, 14 mg DCC and 4 mg NHS are reacted and processed in accordance with example 2.

##### 19. Synthesis of DY - 701

196 mg (0.5 mmol) 7-diethylamino-2,3-dimethyl-4-phenyl-chromenylium-tetrafluoroborate and 242 mg (0.5 mmol) 3-(3-ethoxycarbonylpropyl)-2,3-dimethyl-5-sulfonato-1-(3-sulfonatopropyl)-3*H*-indolium sodium salt are reacted and processed in accordance with example 1.

150 mg (39 %) yield - UV/Vis (Ethanol)  $\lambda_{\text{max}}$  ( $\epsilon$ ) = 706 nm (115.000 l·mol<sup>-1</sup>·cm<sup>-1</sup>). - fluorescence  $\lambda_{\text{em}}$  = 731 nm. - MS (ESI<sup>+</sup>): 747.2 [M]<sup>+</sup>; 373.4 [M-H]<sup>2+</sup>. - C<sub>39</sub>H<sub>43</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>Na (770.90).

## 20. Synthesis of DY – 701 N-Hydroxysuccinimide ester

15 mg DY - 701, 14 mg DCC and 4 mg NHS are reacted and processed in accordance with example 2.

## 21. Synthesis of DY - 781

180 mg (0.5 mmol) 4-*tert*-butyl-7-diethylamino-2-methyl-chromenylium-tetrafluoroborate and 307 mg (0.5 mmol) 3-(3-ethoxycarbonylpropyl)-3-methyl-2-(4-phenylaminobuta-1,3-dienyl)-5-sulfonato-1-(3-sulfonatopropyl)-3*H*-indolium sodium salt are made to react and processed in accordance with example 11.

125 mg (33 %) yield - UV/Vis (Ethanol)  $\lambda_{\max} (\epsilon) = 783 \text{ nm} (98.000 \text{ l}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1})$ . – fluorescence  $\lambda_{\text{em}} = 800 \text{ nm}$ . – MS (ESI<sup>+</sup>): 785.3 [M + Na]<sup>+</sup>; 763.3 [M + H]<sup>+</sup>; 404.4 [M + 2Na]<sup>2+</sup>; 393.5 [M + H + Na]<sup>2+</sup>. – C<sub>38</sub>H<sub>47</sub>N<sub>2</sub>O<sub>9</sub>S<sub>2</sub>Na (762.92).

## 22. Synthesis of DY – 781 N-Hydroxysuccinimide ester

15 mg DY - 781, 14 mg DCC and 4 mg NHS are reacted and processed in accordance with example 2.